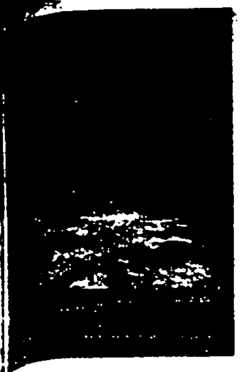
Exhibit 2



nature

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Soviet and French teams working through difficult winter conditions have completed a record of the effects of last year's Armenian earthquake. For the devastation and deaths caused, the 'quake was surprisingly small. Cover shows a surface fault with Spitak in the background. See page 675.

THIS WEEK

usion exchanges

amma rays from cold fusion? ere are two schools of thought. ge 667. And on page 690, a assessment of calculated fusion tes in isotopic hydrogen mol- ules concludes that a 5 to 10- d enhancement of electron ass is required to bring calcu- ted cold fusion rates up to the ge of values claimed experi- entally. The authors "know of plausible mechanisms for hieving such enhancement".

Money grows on trees

he debate about the destruction tropical rainforests assumes e question to be one of econ- my versus ecology. But the ue of products such as fruit d latex, which do not involve forestation, may be two to ee times higher than that of mber exports. Commentary, ge 655.

inase connections

link between cell-cycle control d the regulation of transcrip- on is provided by the discovery at the mouse kinase that phos- orylates a repetitive domain of NA polymerase is encoded by the ouse homologue of cell-cycle ntrol gene *cdc2*. Page 679.

opper counts

the high-temperature super- nductor $Bi_2CaSr_2Cu_2O_8$, it is the pper oxide planes that contain e 'Fermi liquid' electronic ates central to superconductiv- the BiO plane is non-metallic. ge 691.

xtinction patterns

rope has far fewer tree species an eastern Asia, where the gher species diversity may be e to lower rates of glacial ex- ction. The role of factors such long periods of isolation may less important than often sup- sed, page 699.

Coded messages

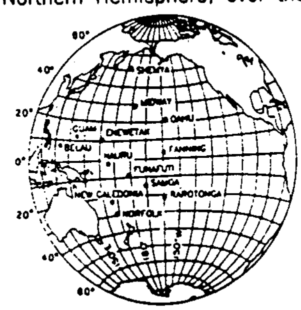
The magnetically disordered materials known as spin glasses constitute a model for a new form of error-correcting code in information transmission. Decoding a signal is equivalent to finding the ground state of the spin glass, and can result in uniquely low error probabilities, pages 693 and 662.

Altering enzymes

A molecular model of the complex formed between tissue-type plasminogen activator (t-PA) and its physiological inhibitor, based on the known three-dimensional structure of a trypsin-inhibitor complex, has facilitated the engineering of an inhibitor-resistant t-PA. Pages 721 and 658.

Pacific atmospheres

Although industrial processes and the burning of fossil fuels have led to large increases in sulphur dioxide emissions in the Northern Hemisphere, over the



Pacific it is biologically produced compounds, mostly dimethyl sulphide from the ocean, that are the main influence on atmospheric sulphur levels and the climatic consequences. Page 685. Pacific nitrate levels, however, seem mainly dependent on anthropogenic nitrate production from continental America, page 687.

Guide to authors

Facing page 726.

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TABLE 1 PCR primers

Region	Name	Sequence 5'-3'	Position	Ref.
C_γ	STP. 120	CTTATGGAGATTTGTTTCAGC	139-145	1
$V_{\gamma 2}$	STP. 121	CGGCAAAAAACAAATCAACAG	37-43	1
$V_{\gamma 4}$	STP. 073	TGTCCTTGCAACCCCTACCC	49-56	29
$V_{\gamma 5}$	STP. 094	TGTGCACTGGTACCAACTGA	35-42	23
$V_{\gamma 6}$	STP. 107	(GGAA)TTCAAAAGAAAACATTGTCT	55-62	23
$V_{\gamma 7}$	STP. 102	AAGCTAGAGGGGTCCTCTGC	18-24	30
C_δ	STP. 110	CGAATTCCACAATCTTCTTG	158-165	3
$V_{\delta 1}$	STP. 111	(GGA)ATTCAGAAGGCAACAATGAAAG	79-86	4
$V_{\delta 3}$	STP. 119	TTCCTGGCTATTGCCTCTGAC	65-72	19
$V_{\delta 4}$	STP. 075	CCGCTTCTCTGTGAACTTCC	61-68	18
$V_{\delta 5}$	STP. 082	CAGATCCTTCCAGTTCATCC	42-49	18
$V_{\delta 6}$	STP. 113	TCAAGTCCATCAGCCTTGTC	72-78	3
$V_{\delta 7}$	STP. 076	CGCAGAGCTGCAGTGTAACT	18-25	18

The position of the nucleotide sequence of the primer is indicated by the corresponding amino-acid number counted from the putative N-terminal cleavage site in each reference. For the $V_{\delta 6}$ primer, a sequence common to pλ12, Z53 and Z49 was chosen. The 3' 15 bases of this primer are also common to M23 (ref. 4).

The amino-acid sequences deduced from the junctional nucleotide sequences indicate that IEL γδ TCR would have a high degree of structural diversity in the *V-J* junctional regions (data not shown); but diversity is not limited to these regions because the $V_{\gamma 7}$-coded γ-chain can pair with either the V_4, V_5, V_6 or V_7 δ-chain. This diversity of the IEL γδ TCR is reminiscent of that observed for the γδ TCR expressed on the thymocytes of adult mice[18,19]. The IEL γδ TCR, however, clearly comprise a unique subset distinct from those on adult thymocytes which use $V_{\gamma 4}$ and $V_{\delta 5}$ gene segments predominantly.

The γδ TCR expressed on DEC, the other known epithelium-associated γδ T-cell subset, utilize a single V_γ ($V_{\gamma 5}$) and a single V_δ ($V_{\delta 1}$) gene segment and have no junctional diversity[14]. This suggests that the ligand for DEC γδ TCR is monomorphic unlike those of αβ TCR[14]. By contrast, IEL certainly have the capacity to recognize structurally diverse ligands with their highly diverse γδ TCR. This, plus the fact that IEL are CD8-positive[20,21] strongly suggests that their ligand is composed of a structurally variable peptide presented by a class I or class I-like protein of the major histocompatibility complex (MHC). The high level of diversity concentrated in the *V-(D)-J* junctions is consistent with the recognition of variable peptides, if the folding of polypeptide chains is similar for TCR γδ and immunoglobulin molecules[22]. The origin of the postulated peptides is a matter of speculation. One possibility is that they originate from a relatively large set of self proteins whose syntheses are induced when the epithelial cells are under stress. Another possibility is that the peptides arise from viruses, bacteria and other microorganisms that are prone to infect the intestinal epithelium cells. The preferential usage of the $V_{\gamma 7}$ segment may reflect its affinity for a limited number of class I or class I-like protein(s) that may be expressed on intestinal epithelial cells. □

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Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin

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IN addition to having profound effects on embryonic pattern formation[1-5], retinoic acid (RA) has striking effects on differentiation and maintenance of epithelial cells *in vivo* and *in vitro* (reviewed in refs 6 and 7). Skin is a major target organ for retinoids both in its normal[6-9] and pathological states[10]. The discovery of two human nuclear receptors for RA (hRARα and hRARβ) acting as transcriptional RA-inducible enhancer factors[11-14] has provided a basis for understanding how RA controls gene expression[15,16]. To investigate the specific role that RARs might play during development and in adult tissues, we have cloned the mouse RARα and RARβ (mRARα and mRARβ). Their amino-acid sequences are much more homologous to those of hRARα and hRARβ, respectively, than to each other, which suggests strongly that RAR α- and β-subtypes have different functions. Most interestingly we have discovered a novel RAR subtype (mRARγ) whose expression in adult mouse seems to be highly restricted to skin, whereas RARα and RARβ are expressed in a variety of adult tissues. Furthermore, both mRARα and mRARγ RNAs are readily detected in undifferentiated F9 embryocarcinoma (EC) cells, whereas mRARβ messenger RNA is induced at least 30-fold in RA-differentiated F9 cells.

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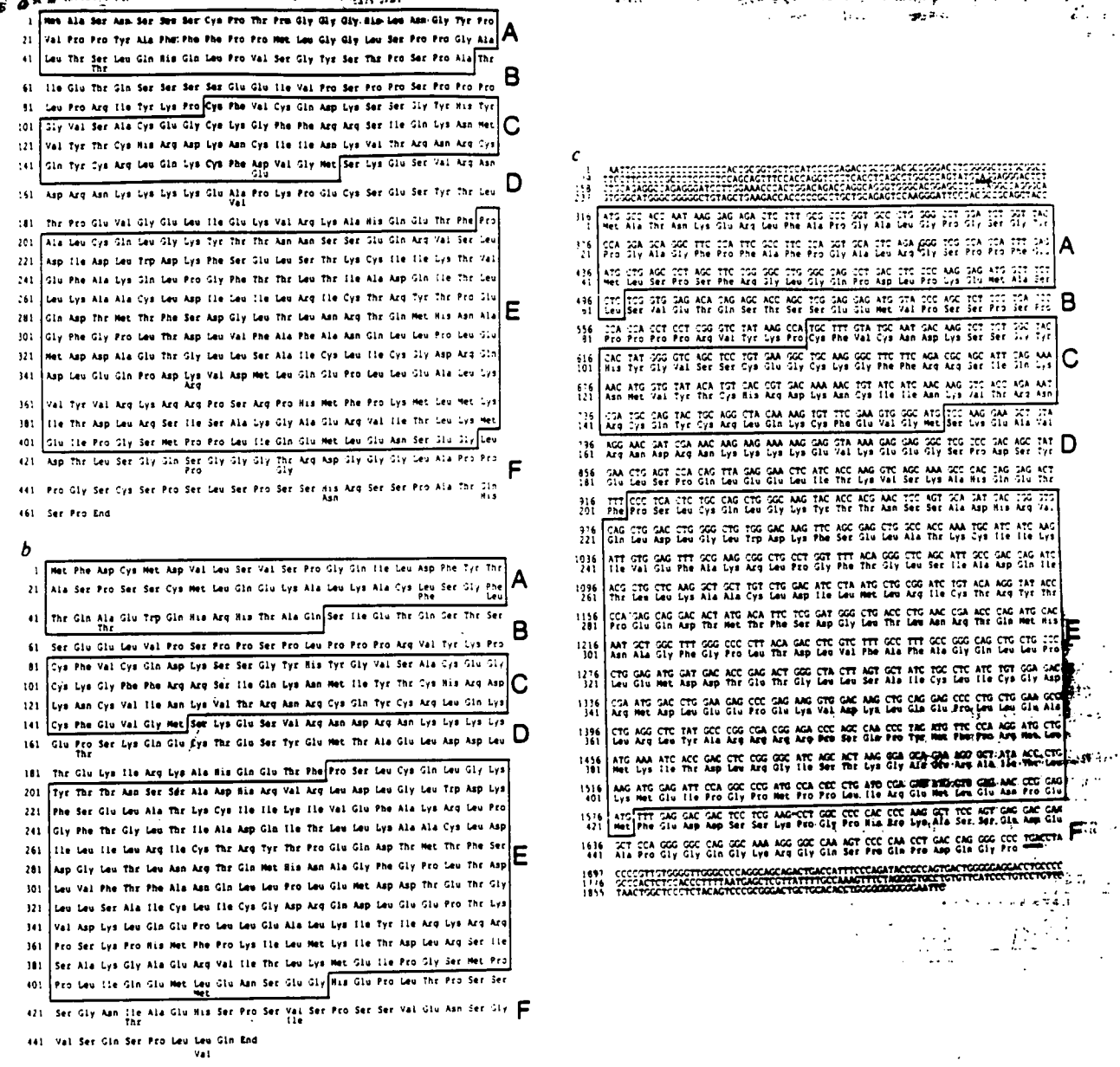

FIG. 1 Amino-acid sequences of mRARα (*a*) and β (*b*), and nucleic-acid and deduced amino-acid sequences of mRARγ (*c*). Nucleotides and amino acids are numbered on the left side of each sequence. An in-frame termination codon (positions 145–147) in the 5′-untranslated region of mRARγ cDNA is underlined in *c*. Regions A, C and E are boxed, and the location of regions A–F is indicated on the right side of each sequence (see text). Amino acids differing between mRARα and mRARβ and their human cognates are indicated in *a* and *b* under the murine sequences. The termination codon at the end of the mRARγ sequence is underlined in *c* and the ends of mRARα and mRARβ amino-acid sequences are indicated in *a* and *b*. The sequences of mRARα and mRARβ cDNAs are available upon request.

METHODS. Approximately 2×10^6 recombinant phage from an 11.5-day, randomly-primed, mouse-embryo λgt10 cDNA library (donated by B. Galliot and D. Duboule) were screened with purified hRARα and hRARβ cDNA probes derived from clones hRARα0 and hRARβ0[11,13] and ^{32}P-labelled by random priming[24] to a specific activity of 10^9 c.p.m. μg^{-1} of DNA. Plaque lifts onto nitrocellulose filters and treatment of the filters before hybridization were carried out as described previously[24]. Hybridization was carried out at 37 °C for ~24 h in 5 × SSPE (0.75 M NaCl, 50 mM NaH_2PO_4 and 5 mM EDTA, pH 7.4), 40% formamide, 0.2 mg ml^{-1} sheared and denatured salmon-sperm DNA, and 1 × Denhardt's reagent[24]. The most stringent wash was done in 2 × SSPE plus 0.1% SDS/0.06% sodium pyrophosphate (NaPPi) at 50 °C for 20 min. Filters were exposed for 36 h at −80 °C (one intensifying screen, Kodak XRO-5 film). Positive clones were rescreened under the same conditions and phage DNA was prepared from the clones which remained positive. These clones were divided into three groups based on Southern blot hybridization data with hRARα and hRARβ cDNA probes and restriction enzyme digest patterns. Selected cDNAs from each group were subcloned into either pTZ19R or pEMBL18 and 19 vectors; single stranded DNA was prepared[24], and sequenced using the dideoxy chain-termination procedure[25]. Two clones which contained the entire open reading frame of mRARγ and mRARβ were sequenced either on both strands (mRARγ, sequence shown in panel *c*), or on one strand (mRARβ, panel *b*; any ambiguity was resolved by sequencing on the other strand). The mRARα sequence was derived from two clones which were sequenced on both strands and overlapped between amino acids 64 and 283 of the sequence displayed in panel *a*. A mRARα cDNA clone containing the entire open reading frame was subsequently constructed in pSG5 (ref. 21) by using the unique *Bsu*36I site located in the DNA sequence encoding amino acids 182–184.

An 11.5-day-old total mouse embryo λgt10 complementary DNA library was screened with hRARα and hRARβ cDNA probes: 81 clones were isolated, of which two sets were identified as mRARα and mRARβ on the basis of a 98% homology of their cDNA-deduced amino-acid sequence with that of hRARα and hRARβ, respectively (Fig. 1*a*, *b*). Less homology with RARα or RARβ was found for a third set of clones, although the deduced amino-acid sequence (Fig. 1*c*) was obviously related to both of them (Fig. 2; the A–F regions in Figs 1 and 2 were as previously defined in refs 11, 13 and 16). This new member of the mouse RAR subfamily was designated mRARγ. The greatest amino-acid sequence similarities among the three mRARs were found in the regions corresponding to the DNA-binding domain (region C, 95%) and the ligand-binding domain (region E, 85% identity between mRARα and mRARγ, and 90% identity between mRARβ and either mRARα or γ), suggesting that mRARγ recognizes the same responsive element and binds the same ligand as mRARα and mRARβ (see below). Region B is also conserved (75%, 86% and 79% identity between mRARγ and mRARα, mRARγ and mRARβ, and mRARα and mRARβ, respectively), whereas no conservation was seen in this region when comparing nuclear receptors that bind different ligands (ref. 16 and refs therein). The D region, which is not conserved across the nuclear-receptor family of a given species and may act as a hinge region[16], is less conserved among mRARs. Both the N- and C-terminal segments of region D, however, are highly conserved, although the central segment is not (hatched box in Fig. 2). No significant similarity was found between mRARs in region A (encoded in an exon different from that encoding region B, see ref. 13), nor in region F, both of which also vary within a given species between the different nuclear receptors[16].

By contrast, there is an almost complete conservation of amino-acid sequence between the A regions of hRARα[11,12] and mRARα (98%), and hRARβ[13,14] and mRARβ (94%), and between the corresponding F regions (90% for hRARα and mRARα, and 92% for hRARβ and mRARβ). Similarly, the entire D region of a given RAR subtype is conserved across species (98% identity between both hRARα and mRARα, and hRARβ and mRARβ). This high degree of conservation of the A, D and F regions for a given RAR subtype contrasts with the lack of or lower conservation of the same regions among the various RARs in a given species (see above) and also with the lower conservation of regions A/B, D and F for a given steroid-hormone receptor across species[16,17]. Thus the A, B, D and F regions may have specific functions not performed by the C and E regions, but necessary for the three RARs to exert their specific physiological roles. Note that the A/B regions of the oestrogen and progesterone receptors have been implicated in specific transcriptional transactivation of some target genes[16,18–20].

When mRARα, mRARβ and mRARγ cDNAs were expressed[21] in HeLa cells together with a reporter plasmid $(TRE3)_3$-tk-CAT containing a RA-responsive element (Fig. 3), all three receptors responded similarly to all-trans RA. As was the case for hRARα and hRARβ[11,13], retinol was much less efficient at the same concentrations (data not shown). No obvious difference was observed between the dose responsiveness of mRARα and mRARβ, in contrast with results obtained previously with human chimaeric RARα and RARβ[13]. This may be due to the use of a less sensitive responsive element in the present study.

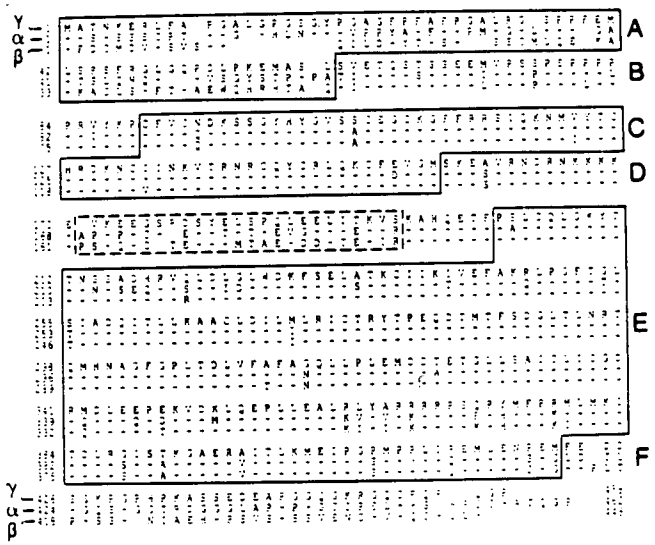

FIG. 2 Amino-acid alignment of the mRARα, mRARβ and mRARγ (as indicated). The single letter amino-acid code is used, and the number of the last amino acid in each sequence is given at the end of the alignment. Numbers on the left side correspond to the first amino acid in a given line. Regions A, C and E are boxed and regions A–F are designated with capital letters on the right side of the figure. The non-conserved part within region D is boxed with a hatched line. Gaps have been introduced to obtain the optimal alignment of the mRARγ sequence with that of mRARα and mRARβ. Dashes represent mRARα and mRARβ amino acids which are identical with those of mRARγ.

FIG. 3 RA-dependent transcriptional trans-activation by mRARs transiently expressed in HeLa cells. HeLa cells were co-transfected as described[11,13] with pSG5-based expression vectors[21] containing the entire cDNA of either mRARα (mRARα0), mRARβ (mRARβ0) or mRARγ (mRARγ0), and a reporter plasmid, $(TRE3)_3$-tk-CAT, carrying a synthetic RA-responsive element (RARE). After transfection (24 h), cells were fed with media containing increasing concentrations of RA as indicated ($0–1\times10^{-6}$ M, lanes 1–8, respectively) and collected 48 h after transfection for determination of CAT (chloramphenicol acetyltransferase) activity.

METHODS. HeLa cells ($\sim10^6$ per dish) were co-transfected with 0.5 μg of a given mRAR expression vector, 2 μg of reporter plasmid and 2 μg of pCH110 (Pharmacia, a β-galactosidase expression vector used as an internal control to normalize for variations in transfection efficiency). The total amount of transfected DNA was adjusted to 20 μg by addition of carrier DNA (BSM13+). Cell culture media, treatment of cells, preparation of cytosolic extracts, and CAT assays were carried out as previously described[11–13]. The reporter plasmid $(TRE3)_3$-tk-CAT (ref. 26) contains a trimer of a synthetic RARE (5′-AGCTTAGGTCAGGGACGTGACCTT-3′) inserted in pBLCAT8[27] (ref. 27) upstream of the tk promoter. mRARα0 was constructed by inserting the reconstructed mRARα cDNA (see Fig. 1) into the *Eco*RI site of pSG5 (ref. 21). mRARβ0 was obtained by inserting the *Eag*1 *Bam*HI fragment of mRARβ cDNA into the *Bam*HI site of pSG5 with the help of a *Bam*HI/*Eag*1 adaptor, and mRARγ0 was constructed by ligating the *Eco*RI-flanked mRARγ cDNA (Fig. 1*c*) into the *Eco*RI site of pSG5.

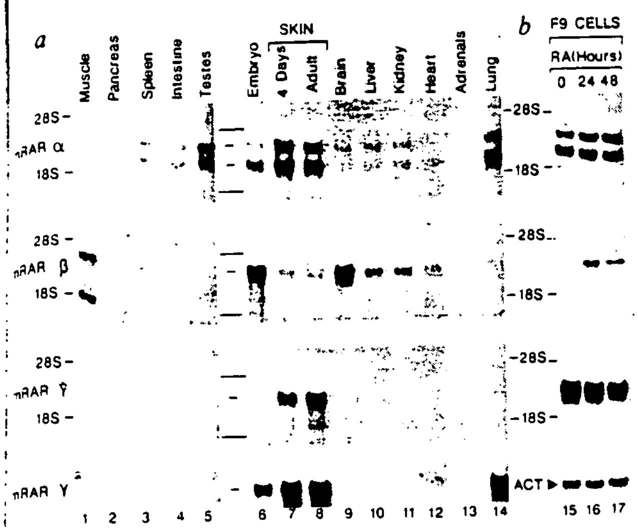

FIG. 4 Northern blot analysis of mRARα, mRARβ and mRARγ poly(A)$^+$ RNA from various mouse tissues (as indicated in *a*, lanes 1-14), and F9 EC cells (*b*, lanes 15-17) before (lane 15), and after 24 h (lane 16) and 48 h (lane 17) treatment with RA (3.3×10^{-7} M). Poly(A)$^+$ RNA (4 μg) was loaded in all lanes. The 28S and 18S rRNA standards were taken as being 4,712 (ref. 28) and 1,869 (ref. 29) bases long, respectively. *a*, mRARα and mRARγ sequences were detected with specific [^{32}P]-end labelled oligonucleotide probes. The entire mRARβ cDNA labelled with [^{32}P] by random priming was used to detect mRARβ RNA. Hybridizations were performed using the same blots, first hybridized with the mRARα probe (exposure time, 7 days at −80 °C; two intensifying screens and Kodak XRO-5 film), then with the mRARβ probe and finally with the mRARγ probe (exposure times, 4 days). The lower panel corresponds to a different blot hybridized only with a randomly primed entire mRARγ cDNA probe (exposure time, 24 h). Actin RNA could be revealed with a cytoskeletal actin cDNA probe[30] in all cases except for pancreas and adrenal preparations suggesting RNA degradation (data not shown). *b*, Three blots were hybridized with randomly primed [^{32}P]-labelled entire mRARα, mRARβ and mRARγ cDNA probes (exposure time, 12 h). The filters were also probed with the actin cDNA probe (lower panel, ACT).

METHODS. RNA was extracted using the GnSCN-CsCl procedure[31]. Poly(A)$^+$ RNA[32] was electrophoresed on 1% agarose-1.1 M formaldehyde gels[33] and transferred to nitrocellulose filters[24]. Hybridisation was as described in the legend to Fig. 1, except that 50% formamide was used and hybridisation was at 42-45 °C (37-40 °C for oligonucleotide probes) for 18 h. Filters were dehybridized by 5-min treatments in 0.05 × SSPE at 90 °C. Specific activity of all randomly-primed cDNA probes and labelled oligonucleotides was $\sim10^9$ and 10^8 c.p.m. μg^{-1} DNA, respectively. The most stringent wash (15 min) was at 65 °C in 0.1 × SSPE plus 1.0% SDS/0.03% NaPPi for filters hybridized with randomly-primed cDNA probes and at 55 °C in 1 × SSPE plus 1.0% SDS/0.03% NaPPi with [^{32}P]-labelled oligonucleotide probes.

The expression of mRARs was investigated using specific oligonucleotide (mRARα and mRARγ) or randomly primed cDNA (mRARβ and mRARγ) probes (Fig. 4*a*). Two mRARα RNAs (~3.8 kilobase (kb) and 2.8 kb) were found in all mouse tissues including skin, and in 11.5-day old embryo (see legend to Fig. 4*a* for pancreas and adrenal). Compared with mRARα RNA, the 3.4-kb mRARβ RNA was relatively more abundant in brain and in total 11.5-day embryo, and lower in skin and lung, than in other tissues. No 3.4-kb mRARβ RNA could be detected in spleen, intestine and testis, and an additional 1.9-kb species was found only in muscle (due to the use of different probes and exposure conditions, the mRARβ signal was amplified at least 20-fold relative to the mRARα signal in Fig. 4*a*). By contrast, it is remarkable that mRARγ RNA was detected at levels at least as high as those of mRARα only in the skin of both 4-day-old and adult animals (upper and third row in Fig. 4*a*). Using a probe of higher specific activity, mRARγ RNA was detectable in total 11.5-day embryo and in lung, and at trace levels in spleen (lower row in Fig. 4*a*). mRARα and mRARγ RNAs were also present in F9 EC cells which differentiate to endodermal-like cells upon exposure to RA[22] (Fig. 4*b*). mRARβ RNA was induced by RA (at least 30-fold from densitometry), whereas no variation was seen for mRARα RNA, and mRARγ RNA decreased by two-fold. A similar induction of mRARβ RNA has been observed by L. Gudas *et al.* (personal communication). Whether this induction is transcriptional, as for hRARβ in a human cell line[23], and is mediated by mRARα or mRARγ or both, is unknown.

In summary, three RAR subtypes are expressed in the mouse, two of them being strikingly homologous to human RARα and RARβ. That hRARα and mRARα, and hRARβ and mRARβ are more homologous to each other than either hRARα and hRARβ, or mRARα and mRARβ, strongly suggests that the three RAR subtypes exert specific functions perhaps by regulating the transcription of different genes at different times of development and in specific cells. In this respect, it is noteworthy that mRARγ expression seems to be highly restricted to skin which is known to be an exquisite RA target in both normal and pathological states[6-10]. Whether mRARγ is specifically expressed in keratinocytes remains to be seen. Finally, the numerous effects of RA on development and the presence of the three RARs in mouse embryo and differentiated F9 cells, raises the question as to whether they exhibit specific patterns of expression and function during embryogenesis. □

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